Inhibition of *N*-methyl-*N*-nitrosourea-induced mutagenicity and DNA methylation by ellagic acid

(DNA alkylation/Ames' *Salmonella typhimurium*/O^6-methylguanine/7-methylguanine)

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ABSTRACT   Ellagic acid, a naturally occurring plant phenol, inhibits the activity of the direct-acting mutagen *N*-methyl-*N*-nitrosourea (MeNU) in *Salmonella typhimurium* TA100. Ellagic acid at 0.10, 0.25, 0.50, and 1.00 mM inhibited the mutagenicity of MeNU (0.40 mM) by 3%, 13%, 45%, and 60%, respectively. Ellagic acid (3 mM) also inhibited the mutagenic activity of *N*,*N*-dimethylnitrosamine (25–200 mM) in the presence of pyrazole-induced rat liver fraction S-9. The effect of ellagic acid on DNA methylation was studied by incubating 0, 0.72, 1.32, 2.64, and 6.60 mM ellagic acid with DNA (0.9 mM nucleotide) and [³H]MeNU (0.66 mM). HPLC analysis of DNA hydrolysates showed that ellagic acid caused a dose-dependent 36–84% decrease in O^6-methylguanine but only a 20% decrease in the 7-methylguanine adduct. Under conditions where methylation at the O^6 position of guanine in double-stranded DNA was inhibited 65% by ellagic acid, no significant inhibition of either O^6- or 7-methylguanine formation was detected in single-stranded DNA. Affinity-binding studies revealed that [³H]ellagic acid binds equally to double-stranded or single-stranded DNA but that poly(dA·dT) binds 1.5 times as much ellagic acid as does poly(dG·dC). The binding of ellagic acid to DNA is dependent on the concentration of both ellagic acid and DNA. The specific inhibition of O^6-methylguanine formation only in double-stranded DNA and the relatively low inhibition of 7-methylguanine formation rule out the possibility that ellagic acid prevents DNA alkylation by scavenging the electrophilic intermediate generated in the hydrolysis of MeNU. The results suggest that ellagic acid inhibition of MeNU-induced mutagenicity is due to specific inhibition of methylation at the O^6 position of guanine through an ellagic acid–duplex DNA affinity-binding mechanism.

Ellagic acid (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3-*cde*][1]benzopyran-5,10-dione), a natural product shikimate derivative present in soft fruits and vegetables (1–3), inhibits the carcinogenicity of benzo[*a*]pyrene (B[*a*]P) (4) and the carcinogenicity and mutagenicity (5–8) and DNA binding (9, 10) of benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), an activated form of B[*a*]P. It was suggested that the chemopreventive activity of ellagic acid results from its ability to accelerate the detoxification of BPDE by forming a BPDE-ellagic acid adduct (11). It was further suggested that the reason for the rapid reaction between BPDE and ellagic acid resulted from an initial π–π nonbonded interaction. The apparent second-order rate constant for the disappearance of BPDE at pH 7.0 is more than 300-fold faster in the presence of ellagic acid (11). In summary, the proposed antimutagenic and antitumorigenic activity of ellagic acid was suggested to result from its direct interception of the "ultimate" electrophilic form of B[*a*]P, thereby preventing DNA adduction.

While this mechanism may be effective with the reactive electrophiles generated in the metabolism of polycyclic aromatic hydrocarbons, it is not likely that such a "scavenging" mechanism would be effective at inhibiting the activity of *N*-nitroso compounds. The central intermediate in the alkylation of DNA by *N*-nitroso compounds is a 1-alkanediazotic acid (12, 13), which is formed directly from the hydrolysis of *N*-nitrosoureas, -carbamates, and -amides or indirectly from the α-oxidation of *N*,*N*-dialkylnitrosamines (Fig. 1). The diazotic acid then ionizes to afford the corresponding alkanediazonium ion (12–14). The transitory alkanediazonium decomposes at extremely rapid rates via a carbonium ion-type transition state (13, 14). Accordingly, this species demonstrates little, albeit some, nucleophilic selectivity (15–17). Thus, for a chemopreventive agent such as ellagic acid to be effective at "reasonable" concentrations against the nonselective nucleophiles generated from *N*-nitroso compounds, it must act with a much higher specificity than can be explained by its scavenging of electrophilic intermediates.

The data presented herein reveal that *N*-methyl-*N*-nitrosourea (MeNU)-induced mutations and *in vitro* DNA methylation, specifically at the O^6 position of guanine, are inhibited by low concentrations of ellagic acid. The prevention of MeNU mutagenicity and DNA binding is discussed in terms of a proposed ellagic acid–duplex DNA affinity-binding mechanism.

MATERIALS AND METHODS

Materials. [³H]MeNU (1.75 Ci/mmol; 1 Ci = 37 GBq) was purchased from Amersham. [³H]Ellagic acid (715 mCi/mmol) was a gift from R. Teel (School of Medicine, Loma Linda University, Loma Linda, CA) or was purchased from Amersham. Both chemicals were radiochemically pure (95–99%) as checked by HPLC or TLC. Nonradioactive MeNU was synthesized by the acid-catalyzed nitrosation of methylurea (Aldrich) and was purified by recrystallization from ethyl acetate/hexane. *N*,*N*-Dimethylnitrosamine (Me₂NNO) was purchased from Aldrich and distilled. Nonradioactive ellagic acid (which was recrystallized from pyridine), salmon sperm DNA (type III, sodium salt), single-stranded (ss) calf thymus DNA (lyophilized powder), yeast RNA (type V), and 7-methylguanine (7-MeGua) were obtained from Sigma. Duplex polydeoxynucleotides (12- to 18-mer) were obtained from Pharmacia P-L Biochemicals. Authentic O^6-MeGua was prepared from 2-amino-6-chloro-purine (Aldrich) (18).

Mutagenicity Studies. MeNU-induced mutations to histidine-independent growth were assessed in *Salmonella*

Abbreviations: B[*a*]P, benzo[*a*]pyrene; BPDE, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; ds, double-stranded; Me₂NNO, *N*,*N*-dimethylnitrosamine; MeGua, methylguanine; MeNU, *N*-methyl-*N*-nitrosourea; ss, single-stranded.
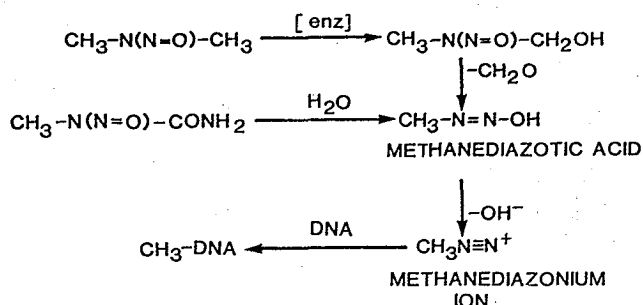*To whom reprint requests should be addressed.

$$CH_3-N(N=O)-CH_3 \xrightarrow{\text{[enz]}} CH_3-N(N=O)-CH_2OH$$

$$\downarrow -CH_2O$$

$$CH_3-N(N=O)-CONH_2 \xrightarrow{H_2O} CH_3-N=N-OH$$
METHANEDIAZOTIC ACID

$$\downarrow -OH^-$$

$$CH_3-DNA \xleftarrow{DNA} CH_3N\equiv N^+$$
METHANEDIAZONIUM ION

FIG. 1. Pathway for the generation of reactive electrophilic intermediates from the enzyme-mediated α-oxidation of Me₂NNO and the hydrolysis of MeNU.

typhimurium strain TA100 as described by Maron and Ames (19). Bacterial suspensions in 0.5 ml of 5 mM potassium phosphate buffer (pH 7.4) were preincubated for 10 min with dimethyl sulfoxide (20 μl), or ellagic acid in dimethyl sulfoxide (20 μl), at final concentrations of 0.10, 0.25, 0.50, and 1.00 mM. At the end of preincubation, 400 nmol of MeNU (final concentration 0.40 mM) was added to the incubation mixture. After 48 hr of incubation, revertants were scored on a Biotran III electronic counter (New Brunswick Scientific). All data are derived from two separate experiments performed in triplicate. The Me₂NNO studies were carried out using essentially the same procedure, except liver fraction S-9 (9000 × g supernatant; ref. 19) from pyrazole-induced (150 mg of pyrazole per kg of body weight daily for 3 days, by i.p. injection) 8- to 10-week-old male Sprague–Dawley rats was included for activation. Final Me₂NNO concentrations of 25, 50, 100, and 200 mM were used in the presence of 8.7 mg of S-9 protein.

Methylation of DNA by [³H]MeNU. Salmon sperm DNA, ss DNA, or polydeoxynucleotide was incubated in 50 mM Tris/HCl buffer (pH 7.4) with [³H]MeNU (10 μCi, 0.66 mM) and dimethyl sulfoxide or ellagic acid in dimethyl sulfoxide (0.72, 1.32, 2.64, and 6.60 mM), in 0.3 ml total volume at 37°C overnight. The overnight incubation resulted in complete hydrolysis of MeNU. The pH of the incubation mixture remained constant during the entire period of incubation.

Isolation of Alkylated DNA. At the end of the incubation period the DNA or polydeoxynucleotide was precipitated using 0.1 volume of 2.5 M sodium acetate and 2 volumes of chilled 95% ethanol. The DNA was allowed to precipitate overnight at −20°C and then centrifuged and washed repeatedly with cold 95% ethanol and acetone until the supernatant was free of radioactivity. The DNA was dried under a nitrogen stream or in vacuo. The total binding to DNA was determined by liquid scintillation counting. DNA was quantitated either fluorimetrically (20) or by absorption at 260 nm.

Hydrolysis of Methylated DNA. MeNU-treated double-stranded (ds) or ss DNA or polydeoxynucleotide was hydrolyzed to purine bases in 0.5 ml of 0.1 M HCl for 20 hr at 37°C. The acid hydrolysate, containing free purine bases, was neutralized by the addition of ammonium hydroxide.

Analysis of Alkylated Purine Bases. Alkylated purine bases were fractionated by HPLC essentially as described by Morimoto *et al.* (21). A 100-μl aliquot of the sample and 5 μl of a mixture containing authentic 7-MeGua and O⁶-MeGua were separated by HPLC [column, 4.6 × 250-mm Altex Ultrasphere ODS (5-μm particles); solvent, isocratic 10% methanol in 50 mM ammonium formate buffer (pH 3.5); flow rate, 1 ml/min; detection, 254 nm]. The retention times of 7-MeGua and O⁶-MeGua were 6.1 and 11.8 min, respectively. The eluate was collected in 0.4-ml fractions and counted in 5 ml of Hydrocount (Baker) scintillation fluid.

Nucleic Acid Binding Assays with [³H]Ellagic Acid. ds DNA (3 mM nucleotide), ss DNA (3 mM), RNA (3 mM), (dG·dC)₁₂–₁₈ (15 mM), or (dA·dT)₁₂–₁₈ (15 mM) was incubated overnight at 37°C in a final volume of 1 ml consisting of 50 mM Tris/HCl buffer (pH 7.4) with [³H]ellagic acid (7 μmol/ml, 715 mCi/mmol). At the end of incubation, the nucleic acid was precipitated and gently washed as described above. The washed and vacuum-dried nucleic acid was dissolved in 1 ml of 50 mM Tris buffer (pH 7.4), and total binding was quantitated by scintillation counting.

Ultraviolet Studies on Ellagic Acid Binding to DNA. Spectra of 3-ml incubation mixtures containing 50 mM Tris buffer (pH 7.4), 0.2–1.0 μmol of ellagic acid, and DNA (3, 10, 15, and 20 μmol) were monitored at 355 nm. The addition of DNA at all concentrations had no effect on the UV absorbance of ellagic acid.

RESULTS

Mutagenicity. MeNU produced a linear mutagenic dose response at concentrations of 0.05 to 0.40 mM (data not shown). The dose-dependent effect of ellagic acid on the mutagenicity of 0.40 mM MeNU is shown in Fig. 2. Ellagic acid at nontoxic concentrations of 0.10, 0.25, 0.50, and 1.00 mM inhibited MeNU-induced mutagenicity by 3%, 13%, 45%, and 60%, respectively. Ellagic acid at the equimolar concentration of MeNU (0.40 mM) caused ≈34% inhibition of mutagenicity. Ellagic acid (3 mM) was very effective in preventing the mutagenic activity of Me₂NNO in the presence of a pyrazole-induced S-9 activating system (Fig. 3). A significant number of revertants above background were only observed in the presence of ellagic acid with 200 mM Me₂NNO. At lower concentrations, no mutagenic activity was observed.

Ellagic Acid Inhibits Methylation of DNA by MeNU. In order to understand the prevention of mutagenicity by ellagic acid, experiments to determine the effect of ellagic acid on DNA methylation were performed. The initial experiments with [³H]MeNU showed that ³H associated with the DNA actually increased with increasing ellagic acid concentrations (data not shown). In these studies the DNA was precipitated with ethanol, centrifuged, and washed repeatedly with ethanol and acetone, and the radioactivity was quantitated by scintillation counting. However, denaturation of the DNA caused a major decrease in DNA-associated ³H (Table 1), which indicated noncovalent binding of ³H, that was assumed to be derived from the affinity binding of methylated ellagic acid to DNA. This was confirmed by preincubating ellagic acid and
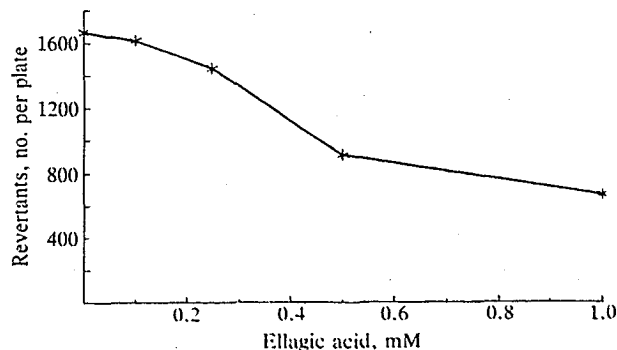
FIG. 2. Ellagic acid decreases MeNU mutagenesis of *S. typhimurium* TA100. Mutagenicity of 0.40 mM MeNU in the absence of ellagic acid was 1666 ± 146 histidine-independent revertants per plate. Mutagenicity of 20 μl of dimethyl sulfoxide (control) was 135 ± 25 revertants per plate. Each value is the mean of two triplicate determinations.
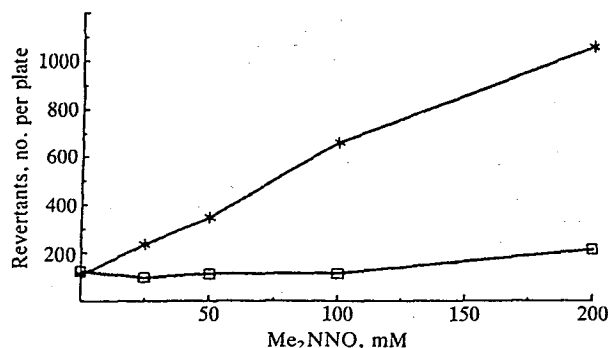
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FIG. 3. Ellagic acid (3 mM) on the mutagenicity of Me_2NNO in *S. typhimurium* TA100 in the presence of pyrazole-induced rat liver fraction S-9 (8.7 mg of protein). Revertants per plate without (×) and with (□) ellagic acid are shown for various concentrations of Me_2NNO. Background mutation rate with 20 μl of dimethyl sulfoxide was 124 ± 15 revertants per plate. Each data point represents the mean of two triplicate determinations.

[³H]MeNU overnight in buffer and then adding DNA. The DNA was isolated and washed, and the binding was as high as when DNA alone was incubated with MeNU (Table 1). We do not know whether the ellagic acid in incubation 2 (Table 1) was methylated while it was noncovalently bound to DNA and/or whether methylation took place in solution and then the methylated ellagic acid bound to DNA.

More detailed studies to determine the effect of ellagic acid on DNA methylation by MeNU were conducted (Table 2). There appears to be a threshold concentration below which ellagic acid has no effect. However, above 0.72 mM ellagic acid, a marked, dose-dependent (40% to 85%) inhibition of O^6-MeGua formation was observed, while only a 20% decrease in the 7-MeGua adduct was seen at all ellagic acid concentrations tested above 0.72 mM. The inhibitory effect of ellagic acid was also observed with $(dG\cdot dC)_{12-18}$ (Table 3). Consistent with a noncovalent binding hypothesis, no inhibition was observed in the MeNU-mediated methylation of ss DNA (Table 3).

Binding of Ellagic Acid to DNA. Attempts to perform competitive binding studies using equilibrium dialysis were unsuccessful because ellagic acid bound to the dialysis tubing. A series of experiments was then conducted to

Table 1. Radioactivity associated with DNA after incubation with [³H]MeNU in the absence or presence of ellagic acid

Conditions	Covalently bound [³H]Me, pmol/μmol of nucleotide	
	Before denaturation	After denaturation*
Incubation 1†		
Control	280 ± 67	127 ± 3
Ellagic acid	2126 ± 333	460 ± 3
Incubation 2‡		
Control	913 ± 197	483 ± 57
Ellagic acid	1296 ± 17	1137 ± 167

Each value is the mean of three determinations ± standard deviation.
*DNA was denatured by heating on a steam bath for 15 min.
†[³H]MeNU (0.2 mM, 1 μCi) was incubated ≈16 hr with dimethyl sulfoxide (20 μl) without (control) or with 0.2 mM ellagic acid at 37°C in Tris buffer (pH 7.4). Salmon sperm DNA (0.9 mM nucleotide) was then added and the incubation was continued ≈16 hr at 37°C.
‡DNA (0.9 mM nucleotide) was incubated with dimethyl sulfoxide without (control) or with ellagic acid for 60 min at 37°C in Tris buffer (pH 7.4). MeNU (0.2 mM, 1 μCi) was added and incubation was continued ≈16 hr at 37°C.

Table 2. Effect of ellagic acid on methylation of DNA by [³H]MeNU

Ellagic acid, mM	DNA adducts, pmol/μmol of nucleotide		O^6/N-7 ratio
	7-MeGua	O^6-MeGua	
0	103.3 ± 4.0	20.0 ± 4.3	0.19
0.72	111.5 ± 4.3	20.1 ± 4.0	0.18
1.32	82.6 ± 3.2	12.7 ± 2.7	0.15
2.64	83.6 ± 3.2	7.1 ± 1.5	0.08
6.60	84.6 ± 2.6	3.3 ± 0.3	0.04

Incubation mixture (300 μl) contained 50 mM Tris buffer (pH 7.4), [³H]MeNU (0.66 mM, 10 μCi), salmon sperm DNA (0.9 mM nucleotide), and dimethyl sulfoxide with ellagic acid at final concentrations as indicated. The mixture was incubated ≈16 hr with gentle shaking at 37°C. Each value is the mean of three determinations ± standard deviation.

address the noncompetitive affinity binding of ellagic acid to DNA and to determine whether there was any preference for G·C versus A·T base pair sequences or ss versus ds DNA. Fig. 4a shows the data on the binding of [³H]ellagic acid to calf thymus DNA at various concentrations of [³H]ellagic acid. Ellagic acid binding to DNA was linear between 0.5 and 3.4 mM ellagic acid. At equimolar (4.8 mM) concentrations of both ellagic acid and DNA, the binding was extrapolated to be 85 pmol of ellagic acid per μmol of nucleotide. Fig. 4b shows the ellagic acid binding to DNA at various concentrations of DNA. Binding was linear between 0 and 8 mM DNA (nucleotide).

Table 4 shows the noncompetitive binding of [³H]ellagic acid with various DNAs, including ds DNA, poly(dG·dC) and poly(dA·dT), and ss DNA. There was no significant difference in binding between ds and ss DNA. Poly(dA·dT) bound 1.5-fold more ellagic acid than did poly(dG·dC), indicating that ellagic acid should have a preference for A+T-rich sites in DNA. UV studies on the interaction of ellagic acid with DNA showed no change in absorbance or UV maximum of ellagic acid at 355 nm with increasing concentrations of DNA (results not shown). It should be pointed out that the [³H]ellagic acid used in the above affinity-binding studies contained ≈5% radioactive impurity. However, UV quantitation of DNA affinity binding with pure nonlabeled ellagic acid showed the same results, but the studies could only be carried out at the higher concentrations of ellagic acid because of the lower sensitivity of the method.

DISCUSSION

N-Nitroso compounds (N,N-dialkylnitrosamines, N-alkyl-N-nitrosoureas, etc.) comprise a class of carcinogens possibly involved in the etiology of human cancer. It has been suggested that mutagenicity and carcinogenicity of N-nitroso

Table 3. Effect of ellagic acid on MeNU-mediated methylation of ds and ss DNA and poly(dG·dC)

Substrate	Ellagic acid	DNA adducts, pmol/μmol of nucleotide		O^6/N-7 ratio
		7-MeGua	O^6-MeGua	
ds DNA	−	231.3 ± 26.6	25.6 ± 2.0	0.11
	+	178.6 ± 20.0	10.6 ± 0.7	0.05
ss DNA	−	76.6 ± 26.0	9.3 ± 1.6	0.12
	+	84.0 ± 10.6	12.0 ± 2.0	0.14
$(dG\cdot dC)_{12-18}$	−	120.3 ± 10.0	8.7 ± 0.3	0.07
	+	80.0 ± 6.0	3.7 ± 0.2	0.04

[³H]MeNU concentration was 0.66 mM (10 μCi). Ellagic acid, when present (+), was at a concentration of 1.3 mM. Each value is the mean of three determinations ± standard deviation.
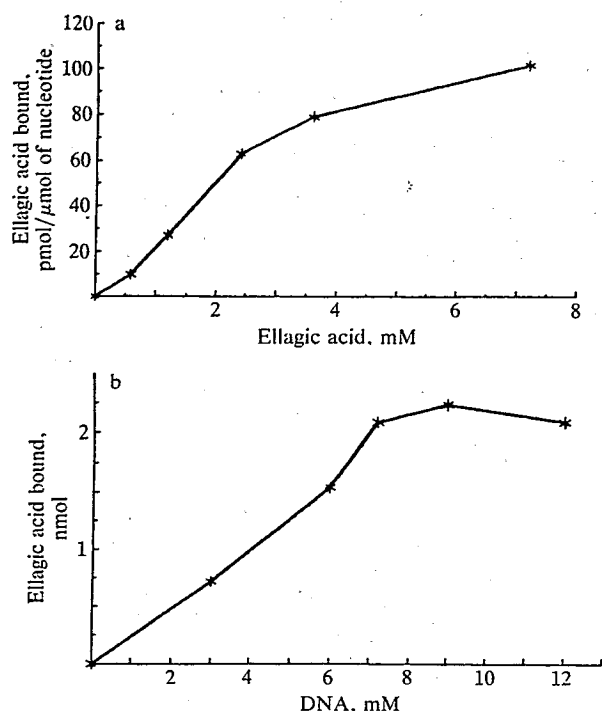
FIG. 4. Binding of ellagic acid to DNA. (*a*) In a final volume of 1 ml were incubated DNA (4.8 mM nucleotide) and various concentrations of [³H]ellagic acid in 50 mM Tris/HCl buffer (pH 7.4). Binding was quantitated as described in *Materials and Methods*. Each value is the mean of three replicate determinations. (*b*) Binding was assayed as in *a*, except that ellagic acid concentration was 6.6 mM and DNA concentration was varied. Each value is the mean of three determinations.

compounds are related to their ability to alkylate DNA (22, 23). Since the initial study by Loveless (24), several lines of evidence indicate that O^6-alkylguanine is one of the critical lesions in DNA that is associated with mutagenesis and carcinogenesis by *N*-nitroso compounds (25–30).

The present study demonstrates that ellagic acid at low and nontoxic concentrations (*i*) inhibits the mutagenicity of MeNU and Me₂NNO, (*ii*) inhibits the MeNU-mediated methylation of guanine in DNA at the O^6 position without significantly altering methylation at the N-7 position, and (*iii*) inhibits the MeNU-mediated methylation of guanine at the O^6 position only when ds DNAs are used as substrate. These results indicate the involvement of a very different mechanism than that proposed for the ellagic acid-mediated prevention of BPDE mutagenicity and carcinogenicity (11). In the BPDE work, data were presented that are consistent with an efficient scavenging of electrophilic BPDE by ellagic acid. Our data indicate a mechanism dependent on ellagic acid–duplex DNA binding being responsible for the prevention of MeNU-mediated mutagenicity and DNA methylation.

Table 4. Noncompetitive binding of [³H]ellagic acid to nucleic acids

Nucleic acid	Ellagic acid bound, $pmol/\mu mol$ of nucleotide
ds DNA (3 mM)	1233 ± 57
ss DNA (3 mM)	1133 ± 33
$(dG\cdot dC)_{12-18}$ (15 mM)	1866 ± 260
$(dA\cdot dT)_{12-18}$ (15 mM)	2733 ± 533
RNA (3 mM)	1533 ± 57

Ellagic acid concentration was 7 mM. Each value is the mean of three determinations ± standard deviation.

It has previously been shown that spermidine, which binds to A·T sites in DNA, can inhibit methylation of rat liver chromatin in a dose-dependent way (31). In those studies MeNU was the methylating agent and 90% inhibition occurred with 0.10 mM spermidine and 2.0 mM MeNU in the presence of 0.6 mM (nucleotide) chromatin or DNA. Under the same conditions, 0.0125 mM spermidine gave 25% inhibition of methylation. In the same incubation mixture the methylation of 2′-deoxy-5′-guanylic acid was not affected by spermidine. The interpretation of these data was that the spermidine inhibition is specific for duplex DNA because of its unique structure. Distamycin A, a DNA minor-groove binder that has an affinity for A+T-rich sites (32), also inhibited guanine methylation in a dose-dependent manner (31). This antibiotic was half as effective as spermidine. It is interesting that an A·T binder such as distamycin A would affect the alkylation of guanine as strongly as it does. However, it is known that distamycin can tolerate isolated G·C base pairs, particularly near the end of its binding site (33). For both spermidine and distamycin A, alkylation at the O^6 and N-7 positions of guanine was monitored, and the decrease in alkylation was equal for both sites. In contrast, ellagic acid selectively inhibits methylation of the O^6 position. The mode of ellagic acid binding to DNA has not been fully characterized. It does not appear to intercalate, based on the failure to observe a hypochromic shift in the UV absorption spectrum of ellagic acid in the presence of DNA (34), and it does show some preference for A+T-rich sequences.

The specific blocking of O^6 guanine methylation by ellagic acid is the first report to our knowledge in which an inhibitor has been shown to diminish specifically the formation of an O^6-alkylguanine adduct. Our data at this time are limited to events occurring at the O^6 and N-7 positions of guanine. Both of these sites face into the major groove. However, the minor-groove binders distamycin and spermidine also inhibit methylation at these two major groove sites (31). It is possible that ellagic acid may indirectly inhibit the methylation reaction by altering the conformation of DNA near its binding site(s) so as to make the major groove O^6 atom less accessible to the methylating species. Another possibility is that when ellagic acid binds to DNA, it is positioned in such a way as to efficiently scavenge reactive intermediate that approaches the O^6 position of guanine. We do not know how ellagic acid–DNA binding might affect methylation at other nucleophilic positions in DNA.

Based on the proposed mechanism of action, ellagic acid should be effective at preventing the mutagenicity of *N,N*-dialkylnitrosamines. In fact, we have found that ellagic acid almost completely inhibits the mutagenicity of Me₂NNO in *S. typhimurium* TA100 in the presence of pyrazole-induced rat liver S-9 (Fig. 4). This inhibition was observed at Me₂NNO concentrations ranging from 25 to 200 mM with 3 mM ellagic acid. In the absence of ellagic acid, a linear mutagenic dose response to Me₂NNO was observed, with approximately 1100 revertants scored at the highest Me₂NNO concentration. Ellagic acid had no effect on Me₂NNO metabolism as measured by formaldehyde production (data not shown).

The results of the present study, which indicate the potential use of ellagic acid as a clinical chemopreventive agent and as a mechanistic tool to probe the biological significance of specific DNA adducts, clearly justify continued research on ellagic acid.

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